

Ultrasensitive Detection of DNA Sequences in Solution by Specific Enzymatic Labeling

Alonso Castro,* Diego A. R. Dalvit, and Lorraine Paz-Matos

Physics and Theoretical Divisions, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

We present a newly developed technique for the direct detection of very low concentrations of specific nucleic acid sequences in homogeneous solution based on a polymerase extension reaction. This method consists of synthesizing a highly fluorescent nucleic acid reporter molecule using a sequence of the target as a template. Synthesis of the reporter molecule is accomplished by hybridizing a short complementary oligonucleotide primer to the target and extending the reporter using a polymerase and free nucleotides. One of these nucleotides is partially labeled with a fluorophore. The reaction sample is then flowed through the capillary cell of a single molecule detector. Detection of the reporter signifies the presence of the target being sought. Under carefully selected conditions, fluorescence from the reporter molecule is much stronger than that of the free nucleotide background over the detection time. We have derived practical equations that allow us to determine an optimal range of values for the relative reporter and free-nucleotide concentrations. This method allows for the rapid, direct detection of individual targets at femtomolar concentrations without the use of an amplification procedure, such as the polymerase chain reaction.

The detection of specific sequences of DNA or RNA with high sensitivity is of fundamental importance in many fields, including genetic and medical research, clinical chemistry, and forensic science, among others. The most widely method used for the identification of specific DNA sequences is the Southern blot. In this procedure, the DNA sample is cleaved with a restriction enzyme, size-separated by gel electrophoresis, and transferred from the gel to a nitrocellulose filter. A sequence-specific probe—usually labeled with a radioisotope—is added to the filter, and the target fragment is then detected by autoradiography. Despite its popularity, Southern blotting suffers from some limitations, mainly because it involves a series of manually intensive procedures that cannot be run unattended, cannot be readily automated, and are susceptible to poor quantitative accuracy and poor reproducibility. The sensitivity problem has been alleviated by the development of techniques for enzymatic amplification of the target sequence prior to analysis. The polymerase chain reaction (PCR), for example, selectively increases the concentration of the target sequence relative to unrelated sequences, thus enhancing

both the specificity and sensitivity of the assay. However, amplification methods may introduce ambiguities from contamination, from variability in amplification efficiency, and from other mechanisms not yet fully understood.^{1–7} There is, therefore, the need for the development of new methods for the detection of specific nucleic acid sequences bearing the following desirable characteristics: (1) high sensitivity: single target molecule detection, which circumvents the use of enzymatic amplification reactions; (2) high specificity: the ability to detect a short sequence in the presence of unrelated sequences from a whole genome; (3) homogeneous assay format: permitting the use of simple reaction protocols, which are amenable to automation; and (4) high throughput: thereby obtaining the desired statistical confidence of detection in a short period of time.

Some of the most promising techniques for achieving this goal rely on the laser-based detection of fluorescent molecules at the single-molecule level of sensitivity. Single-molecule detection has been extensively applied to the detection and sizing of DNA fragments.^{8–11} However, the detection of a specific sequence within a single fragment in homogeneous solution requires the use of hybridization probes, and efficient methodologies for discriminating between the signals from bound and unbound probes must be devised.^{12–15} Fluorescence correlation spectroscopy (FCS), for example, has been applied to the observation of hybridization of single nucleic acid complements by measuring differences in diffusion constants.^{13,14} Since diffusional FCS relies on observing molecular Brownian motion, this method cannot be applied to high-throughput biochemical analysis, which generally requires

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* To whom correspondence should be addressed. Phone: (505)665-8044. Fax: (505)667-1112. E-mail: acx@lanl.gov.

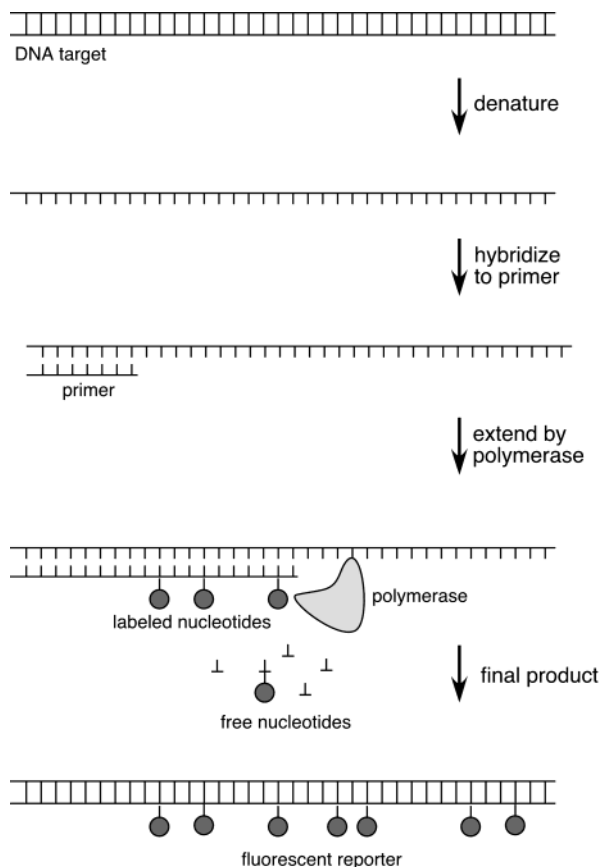


Figure 1. Schematic representation of the polymerase extension reaction for fluorescent reporter molecule synthesis.

fast-flowing solutions. Another method makes use of a new class of DNA probes that show increased fluorescence yields upon hybridization.¹⁵ In this case, multiparameter analysis of single-molecule fluorescence bursts allows discrimination between target-probe complexes and unbound probes. This method has been demonstrated to the study of static solutions, and their application to flowing samples with high throughput should prove useful.

We present here our results for the development of a new method for the detection of specific nucleic acid sequences by using a polymerase extension reaction. Our approach is based on detecting the presence of a specific nucleic acid sequence of bacterial, human, plant, or other origin. The nucleic acid sequence may be a DNA or RNA sequence and may be characteristic of a specific taxonomic group, a specific physiological function, or a specific genetic trait.

This method consists of synthesizing a highly fluorescent nucleic acid reporter molecule using a sequence of the target as a template (Figure 1). A short oligonucleotide primer complementary to the target is added to the sample along with a suitable polymerase and free nucleotides. One of these oligonucleotides is partially labeled with a fluorophore. If the target is present in the sample, the primer binds to it, and the polymerase will incorporate the labeled and unlabeled nucleotides, reconstructing the target's complementary sequence. The sample is then flowed through the capillary cell of a single molecule detector.¹² Detection of the reporter signifies the presence of the target being sought. Since an excess of free nucleotides is needed in order to drive the reaction toward the formation of reporter in a reasonable

amount of time, free labeled nucleotides will remain in the reaction mixture. Under carefully selected conditions, fluorescence from a single reporter molecule will be larger than that originating from free nucleotides over the detection time. It is important, therefore, to make an a priori determination of the optimal values for the relative reporter and free-nucleotide concentrations. To this effect, we have developed simple equations that express the expected signal-to-noise-ratio as a function of reporter and free nucleotide concentrations. This new method has been demonstrated for the detection of a specific sequence of pUC19 DNA, a 2686 base-pair plasmid, in the presence of a large excess of unrelated salmon genomic DNA.

THEORY

The ability to detect the fluorescence signal from a single fluorescent reporter in the presence of a large fluorescence background originating from free labeled nucleotides in a homogeneous solution depends on several factors: the nucleotide incorporation efficiency, the relative initial concentrations of target, primer, and total free probe, and the overall fluorophore detection efficiency. Consider a solution of the reaction product, i.e., the reporter molecule, and unreacted labeled nucleotides contained in the reaction volume \bar{V} . We denote the mean total number of labeled nucleotides contained in that volume as $\langle \bar{N}_T \rangle$ and the mean number of reporter molecules as $\langle \bar{N}_R \rangle$. The mean number of free labeled nucleotides is denoted as $\langle \bar{N}_F \rangle$, whereas the mean total number of incorporated labeled nucleotides is given by $\langle N_B \rangle \langle \bar{N}_R \rangle$, where $\langle N_B \rangle$ is the mean number of incorporated labeled nucleotides per reporter molecule. The solution flowing through the capillary cell is illuminated in the detection volume V by the focused laser beam. In any given time interval Δt , there are N_F free labeled nucleotides in V , their mean number being related to that in the reaction volume as $\langle N_F \rangle \langle \bar{N}_F \rangle = V / \bar{V}$. The concentration of reporter molecules in the reaction volume, $\bar{n}_R = \langle \bar{N}_R \rangle / \bar{V}$, is kept low enough so as to ensure that there are either zero or one reporter molecules in the detection volume at any given instant. At the concentrations used in the present experiments, the probability of having two or more reporter molecules at the same time is negligible. Therefore, for the following derivations, we concentrate on events in which there is indeed only one reporter molecule in V .

The emission from the illuminated volume consists of bursts of fluorescence from labeled nucleotides that are bound to the reporter molecule, from free labeled nucleotides, and from a continuous background due to Raman and Rayleigh scattering and detector dark noise. Under our experimental conditions, the signal due to the continuous background process is much weaker than that from the flowing fluorescently labeled nucleotides, and we will neglect it in what follows.

Fluorescence emission from labeled nucleotides in a free or incorporated state corresponds to two independent, uncorrelated processes. The probability of observing m counts in the measured signal during a time interval Δt is

$$P_s(m, \Delta t) = \sum_{z=0}^{\infty} P_F(z, \Delta t) P_B(m - z, \Delta t) \quad (1)$$

where $P_F(z, \Delta t)$ and $P_B(z, \Delta t)$ are the probabilities of observing z

counts arising from free and incorporated labeled nucleotides in a time interval Δt , respectively. In other words, the observed distribution of counts is a convolution of the fluorescence emission from free and incorporated labeled nucleotides. To calculate these two probabilities we have to consider the probability distribution of fluorescence emission from a single fluorophore and the probability distributions of free and incorporated fluorophores in the volume V . For N fluorophores (either free or incorporated), the probability distribution of emitting z photons in a time interval Δt , $P_N(z, \Delta t)$, is a Poisson distribution

$$P_N(z, \Delta t) = \frac{(\alpha N)^z e^{-\alpha N}}{z!} \quad (2)$$

where α is the mean number of counts from a single fluorophore, assuming that it is the same for bound or unbound fluorophores, and given by $\alpha = \sigma I \eta \Phi \Delta t$. Here σ is the absorption cross-section of a fluorescent molecule, I is the light intensity, η is the photon detection efficiency, and Φ is the photodestruction quantum yield. Since the amount of fluorophores (i.e., labeled nucleotides), N , present in the volume is itself a stochastic quantity, the probability distribution for detecting z photons in an interval Δt is

$$P(z, \Delta t) = \sum_{N=0}^{\infty} P(N) P_N(z, \Delta t) \quad (3)$$

where $P(N)$ is the probability distribution for fluorophores in the volume V . To calculate the probabilities of photodetection given in eq 3 from free and incorporated fluorophores, we need to know the probability distributions $P_F(N)$ and $P_B(N)$ for free and incorporated fluorophores, respectively. For the first one we use the known statistical result that the number of molecules in a small volume communicating with an infinite reservoir is Poisson-distributed; therefore $P_F(N)$ is assumed to be Poissonian. The probability distribution $P_B(N)$ for incorporated fluorophores is unknown. Fortunately for our purposes, we only need its mean, $\langle N_B \rangle$, whose range of values will be determined shortly.

The mean number of counts from free and incorporated fluorophores are

$$\begin{aligned} \langle m_F \rangle &= \alpha \langle N_F \rangle \\ \langle m_B \rangle &= \alpha \langle N_B \rangle \end{aligned} \quad (4)$$

and the standard deviation of the number of counts from free fluorophores is $\sigma_{m_F}^2 = \alpha^2 \sigma_{N_F}^2 + \alpha \langle N_F \rangle$, where the first term, containing the standard deviation in the number of free fluorophores, arises due to the fact that the number of free fluorophores in the volume V is also a stochastic quantity. As we mentioned, its distribution is Poissonian, so that we finally have

$$\sigma_{m_F}^2 = (\alpha^2 + \alpha) \langle N_F \rangle \quad (5)$$

The detection of single reporter molecules is optimized by maximizing the ratio of the signal from bound labeled nucleotides, $\langle m_B \rangle$, to the mean fluctuations of the signal from free labeled nucleotides, σ_{m_F} . Here, we use the generally accepted criterion

that detection is possible if the first signal is at least three times higher than the latter one, namely

$$\frac{\langle N_B \rangle}{\langle N_F \rangle^{1/2}} \geq 3\sqrt{1 + \alpha^{-1}} \quad (6)$$

Using the relation $\langle N_F \rangle / \langle \tilde{N}_F \rangle = V / \tilde{V}$, we obtain the following lower limit for the mean number of bound fluorophores for single DNA fluorescence to be feasible, $\langle N_B \rangle \geq 3\sqrt{(V/\tilde{V})(1+\alpha^{-1})\langle \tilde{N}_F \rangle}$. Using the fact that the mean total number of fluorophores in the reaction volume is given by $\langle \tilde{N}_T \rangle = \langle N_B \rangle \langle \tilde{N}_R \rangle + \langle \tilde{N}_F \rangle$, we can re-express the previous inequality in terms of the concentration of fluorophores $\tilde{n}_T = \langle \tilde{N}_T \rangle / \tilde{V}$, and the concentration of reporter molecules, \tilde{n}_R . It is easy to show that the allowed range of values for $\langle N_B \rangle$ is

$$\langle N_B \rangle_{\min} \leq \langle N_B \rangle \leq \frac{\tilde{n}_T}{\tilde{n}_R} \quad (7)$$

where the minimum value $\langle N_B \rangle_{\min}$ is given by

$$\langle N_B \rangle_{\min} = \frac{9}{2} V (1 + \alpha^{-1}) \tilde{n}_R \left[-1 + \left(1 + \frac{4}{9(1 + \alpha^{-1}) V \tilde{n}_R^2} \tilde{n}_T \right)^{1/2} \right] \quad (8)$$

We can also make an approximation for the case where the mean number of free fluorophores in \tilde{V} is much larger than that of bound fluorophores (i.e., $\langle \tilde{N}_T \rangle \approx \langle \tilde{N}_F \rangle$). In this case, the lower bound for the mean number of fluorophores incorporated to a reporter molecule that permits single reporter molecule detection is

$$\langle N_B \rangle_{\min} \geq 3\sqrt{V(1 + \alpha^{-1})\tilde{n}_T} \quad (9)$$

Equations 8 or 9 allow us to estimate the minimal mean number of fluorophores that must be incorporated into a single reporter molecule so that, when such a molecule is present in the observation volume, the fluorescence signal from the reporter molecule outweighs the signal from the free-fluorophore background. Thus, we have a guidance to help us choose the appropriate parameters for obtaining a successful experimental result. Figure 2 shows a representative graph of eqs 7 and 8.

EXPERIMENTAL SECTION

Sample Preparation. pUC19 DNA Digestion Reaction.

Prior to all experiments, pUC19 DNA (New England BioLabs, Beverly, MA) was digested with the Bgl I restriction endonuclease (New England BioLabs). Two fragments are obtained, 1568 bp and 1118 bp in length. The digestion reaction mixture consisted of pUC19 DNA at a final concentration of 20 ng/ μ L and 5 units of Bgl I in 1 \times NEBuffer 3 (New England Biolabs) in a total volume of 50 μ L. The sample was incubated at 37 $^{\circ}$ C for 1 h, followed by enzyme inactivation at 65 $^{\circ}$ C for 20 min. All digestion and extension reactions were carried out in an automatic thermocycler.

pUC19 DNA Extension Reaction. The reaction mixture consisted of the following: pUC19 DNA digestion product at a

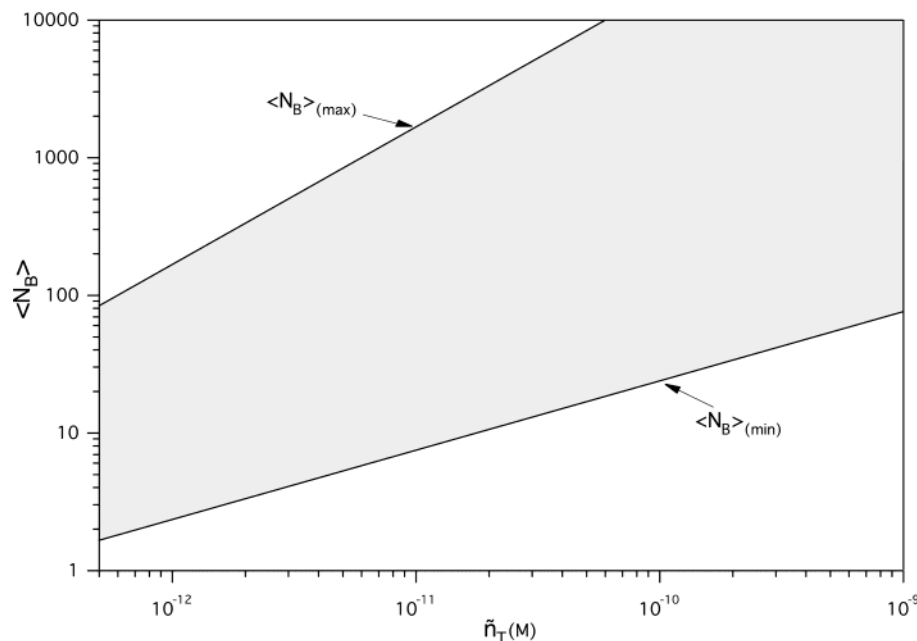


Figure 2. Plot of the minimum and maximum number of incorporated fluorescent nucleotides per reporter molecule vs the total concentration of fluorescent nucleotides, according to eqs 7 and 8, for $\alpha = 20$, $V = 1$ pL, and $\tilde{n}_R = 6$ fM. The shaded area represents the values of $\langle N_B \rangle$ for which the detection of reporter molecules is possible.



Figure 3. Schematic representation of the 1568-base restriction fragment of pUC19 DNA, showing the relative positions of the primer binding site, and the expected 1468-base extension product.

final concentration of 1.2 pM; salmon testes DNA at a 1:1 genomic ratio (2.3 $\mu\text{g}/\mu\text{L}$); a 24-mer pUC/M13 primer (5'-CGCCAGGGT-TTCCAGTCACGAC-3') (Promega, Madison, WI), which binds at positions 352–375 of pUC19 within the 1568-base fragment, at a concentration of 2.5 nM (Figure 3); dATP, dGTP, and dCTP at 500 nM each (Promega); dTTP at 50 nM (Promega); ChromaTIDE Bodipy TMR-14 dUTP at 10 nM (Molecular Probes, Eugene, OR); and 5 units of Klenow fragment of DNA Polymerase I (Fisher Scientific), in 50 μL of buffer solution (50 mM Tris-HCl, 10 mM MgSO_4 , 0.1 mM DTT, pH 7.2). The reaction consisted of denaturation of the target at 95 $^\circ\text{C}$ for 5 min, primer annealing at 58 $^\circ\text{C}$ for 5 min, and extension at 72 $^\circ\text{C}$ for 1 h. A control experiment was performed under identical conditions, except that the pUC19 DNA target was not included. The reaction mixture was then diluted 200-fold in distilled water to yield a 6 fM nominal concentration of target and then analyzed in a single-molecule detector as described below. The relative concentrations of target and labeled nucleotide were chosen following the guidelines established in the Theory section. In the present case, eq 8 yields a value of 11 for the minimum number of fluorescently labeled nucleotides required to allow the detection of reporter molecules, which is well below the expected number of incorporations for the 1468-base extension product.

Instrumentation. A schematic representation of the experimental setup is shown in Figure 4. The excitation source consists of the frequency-doubled output (532 nm) of a mode-locked

Spectra-Physics model 3800 Nd:YAG laser, which produces 70-ps pulses at a repetition rate of 82 MHz. The laser output is attenuated to 5–10 mW and focused by a $6\times$ microscope objective into the 0.5×0.5 mm i.d. square cross-section capillary cell to yield a $10\text{-}\mu\text{m}$ spot. A syringe pump is used to drive the sample through the capillary cell at a rate of 200 $\mu\text{L}/\text{h}$, which translates to an average linear flow velocity of 222 $\mu\text{m}/\text{s}$, and a molecular residence time of 45 ms. The concentration of fluorophores in these experiments is so low that the probability that two molecules occupy the detection volume at a given instant is negligible. Since the fluorescence lifetime of most fluorophores is of the order of a few nanoseconds, a single molecule undergoes thousands of excitation–emission cycles during its travel through the laser beam, which produces a fluorescence photon burst. This fluorescence is collected at right angles by a $40\times$ 0.85 N.A. Nikon microscope objective and spatially filtered by a 0.4×0.4 mm square slit, which defines a 10×10 μm detection area. When combined with the laser excitation cross-section, this detection area defines a detection volume of approximately 1 pL. The light is then spectrally filtered by a 40 nm-bandwidth band-pass interference filter (Omega Optical, RDF series) and detected by an EG&G Canada SPCM-200-PQ single-photon avalanche photodiode (SPAD), which was modified to provide an unamplified raw output, with the purpose of improving the instrumental time response. Despite the use of spatial and spectral filters, some incompletely attenuated excitation light as well as Raman scattering from the solvent reach the detector. These two scattering emissions occur only during the duration of the laser pulse, whereas fluorescence occurs up to several nanoseconds after the excitation pulse. Therefore, by time-resolving the arrival of each photon, scattered light can be rejected, and the majority of the desired fluorescence signal is accepted.¹⁶ The SPAD signal is

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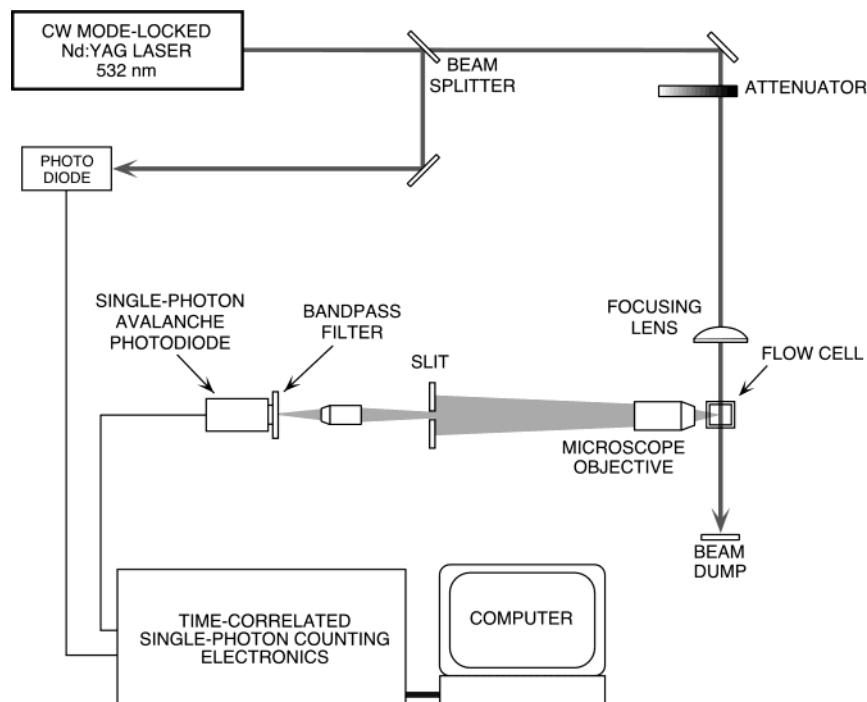


Figure 4. Schematic representation of the experimental setup for single-fluorescent molecule detection.

amplified by a Phillips Scientific 774 amplifier, shaped by a Tennelec TC454 discriminator, and sent to the start input of a Tennelec TC863 time to amplitude converter (TAC). A fraction of the laser beam is split off by a beam splitter and sent to a fast photodiode to provide the stop pulses for the TAC. A time-gate that accepts only delayed photons is set within the TAC. Fluorescence counts are collected in 1 ms intervals by a National Instruments PCI-6111E data acquisition card within a personal computer. Running times per sample are in the 400–800 s range.

RESULTS AND DISCUSSION

The experimental results for the detection of a specific sequence of pUC19 DNA are shown in Figure 5. The top trace corresponds to the pUC19 extension reaction product in the presence of salmon testes DNA. The photon bursts are due to single reporter molecules as they pass through the laser beam. The large, continuous background is due mostly to fluorescence from the excess of unreacted labeled nucleotides. The bottom trace shows the results for the control experiment, i.e., a sample prepared under identical conditions, except that the target pUC19 DNA was not included. No fluorescence bursts were obtained in this case but only the free-nucleotide background. By setting a threshold above the background where no photons are observed in the negative control case and counting the number of peaks observed for the pUC19 sample, the calculated concentration of reporter molecules is 7 fM, in good agreement with the initial concentration of targets. Therefore, we have shown that this technique is suitable for estimating the amount of targets present in a sample. In addition, the mean number of incorporated fluorophores per reporter molecule can be estimated by comparing the mean number of photons per reporter burst to the mean number of photons due to unincorporated fluorophores present in the detection volume during the residence time of a reporter. This estimate assumes that the fluorescence quantum yields for

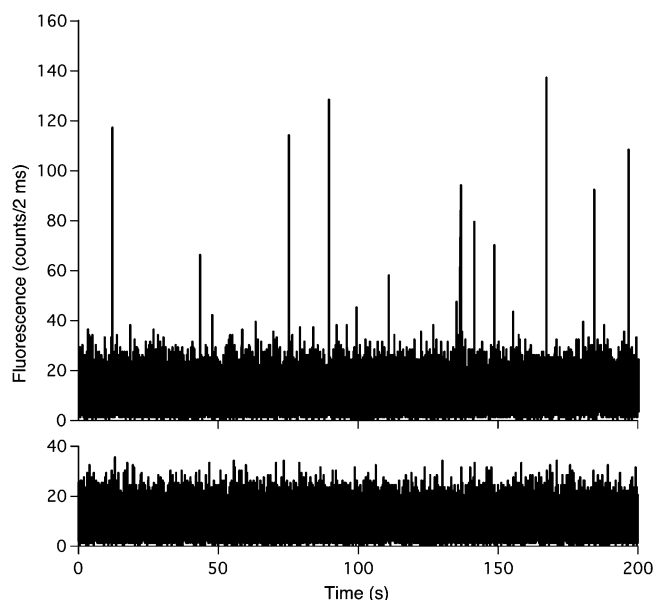


Figure 5. Experimental results for the detection of a specific sequence of pUC19 DNA as a plot of the number of detected counts per 2-ms interval vs time. Top trace: Raw data for the detection of the 1568-bp pUC19 target in the presence of salmon testes DNA. Bottom: Raw data for the negative control, which includes salmon testes DNA but lacks pUC19 DNA.

fluorophores in the bound or unbound states are the same, which is expected given the relatively long 14-member linker arm that attaches the fluorescent tag to the nucleotide. In this way, we obtain a value of 50 for the mean number of incorporated fluorophores per reporter from our experimental data. Since the ratio of unlabeled dTTP to labeled dUTP is 5:1, we expect a nominal maximum number of incorporated fluorophores of 73 for a 1486-base full-extension reporter product size. Therefore, we

conclude that, under the present experimental conditions, the polymerase incorporates fluorescently labeled nucleotides at a relatively high rate and that the extension reaction is probably taking place over most, if not all, of the length of the DNA template.

These results demonstrate the detection of a specific DNA sequence with excellent signal-to-noise ratios (SNR) in the presence of unrelated DNA in as little as 400 s. Since salmon testes DNA was added to the sample at a 1:1 genomic ratio, we have shown that this technique has sufficient specificity to detect a single-copy gene, even for human samples, considering the similar sizes of human and salmon genomes. This high degree of specificity is attributed to the use of a 24-mer complementary oligonucleotide primer. We chose the ChromaTIDE Bodipy TMR-14 dUTP to label our samples because it exhibits good absorption at the excitation wavelength and has well separated excitation and emission profiles, and its incorporation yield by Klenow fragment of Polymerase I is high. We have used relatively low concentrations of labeled dUTP so that the background signal is not excessive, while allowing a nominal maximum incorporation rate of 20% with respect to total dTTP. Under carefully selected conditions, fluorescence from a single reporter molecule is larger than that of the free nucleotide fluorescence background over the detection time, as shown in the present experiments.

A possible strategy to reduce the intensity of the background due to unincorporated labeled nucleotides is to reduce the detection volume, for example by using a confocal optical system for fluorescence detection. This kind of experimental arrangement is widely used in the field of single-molecule detection, and it is mostly useful for observing individual molecules undergoing dynamic processes. However, because of the reduced detection volumes involved in confocal microscopy, namely in the fL range, the analysis throughput of a flowing sample is greatly diminished, making it unsuitable as a practical tool for the bioanalytical laboratory.

A possible enhancement to the present technique is to simultaneously perform "single-molecule electrophoresis"¹⁰ on the

sample. In this method, the electrophoretic mobility of fluorescently labeled molecules (free labeled nucleotides and reporter molecules in this case) can be determined with single-molecule sensitivity. Since single nucleotides exhibit an electrophoretic mobility vastly different to that of nucleic acid targets, interference from free nucleotides can be eliminated even further. This modification to the method will also allow us to simultaneously determine the size of the target, a useful analytical tool for genotyping purposes, for example.

CONCLUSION

The method introduced here allows the rapid detection of specific nucleic acid sequences in solution with high sensitivity and specificity. We have used a simple polymerase extension reaction in homogeneous solution to synthesize fluorescent molecules that report the presence of the a specific target. Judicious selection of the relative concentrations of labeled and unlabeled nucleotides results in the signal of a single reporter molecule to be much larger than that of the free nucleotide fluorescent background present in the detection volume at a given instant. Therefore, there is no need to use a reporter product separation or purification step in the detection procedure. The results presented here are a first step toward the development of powerful, new techniques for the quantitative detection of specific nucleic acid sequences with high sensitivity and specificity. These techniques should allow the analysis of minute amounts of DNA samples directly, i.e., without the need for a target amplification step.

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